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13. ABSTRACT (Maximum 200 words)

The potential of fungal cellulases to release glucose from cellulose in the human gut has been evaluated. Cellulase stability in simulated gastric and intestinal fluids has been investigated and the beneficial effect on stability of neutralizing the gastric fluid (e.g., with antacid) was modeled. Cellulose conversion to soluble sugars by different cellulases under simulated gastric and intestinal conditions was also investigated. Up to 30% conversion of crystalline cellulose is possible under these conditions and over 90% of cellulose hydrolysis can be achieved with other cellulosic substrates. Further evaluation would require better *in vitro* and/or *in vivo* models of the GI tract. We provided a report on controlled-release formulation of enzymes and conclude that there is potential for targeted delivery of enzymes and other functional components (e.g., peptides, probiotics, prebiotics, etc.) to the GI tract. There is potential for enhanced cellulose hydrolysis via a tailored enzyme mix and/or protein engineering to improve enzyme performance. There is also potential for generation of, or release of, "functional" carbohydrates using enzyme treatment of food.

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DARPA BAA03-02 Proposal: Feasibility Assessment For The Use Of Cellulase In Biomass Conversion For Human Application

Contract No: DAAD19-03-C-0113; Proposal No: 45611-LS-000

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Abstract

The potential of fungal cellulases to release glucose from cellulose in the human gut has been evaluated. Cellulase stability in simulated gastric and intestinal fluids has been investigated and the beneficial effect on stability of neutralizing the gastric fluid (e.g., with antacid) was modeled. Cellulose conversion to soluble sugars by different cellulases under simulated gastric and intestinal conditions was also investigated. Up to 30% conversion of crystalline cellulose is possible under these conditions and over 90% of cellulose hydrolysis can be achieved with other cellulosic substrates. Further evaluation would require better *in vitro* and/or *in vivo* models of the GI tract. We provided a report on controlled-release formulation of enzymes and conclude that there is potential for targeted delivery of enzymes and other functional components (e.g., peptides, probiotics, prebiotics, etc.) to the GI tract. There is potential for enhanced cellulose hydrolysis via a tailored enzyme mix and/or protein engineering to improve enzyme performance. There is also potential for generation of, or release of, “functional” carbohydrates using enzyme treatment of food.

Summary of Results

Genencor International's role within the GUT Seedling project (DARPA BAA03-02 Proposal: Feasibility Assessment For The Use Of Cellulase In Biomass Conversion For Human Application) was to evaluate the addition of enzymes to food in order to improve the utilization of (hemi)cellulosic materials for human nutrition.

Genencor's deliverables for this project were as follows.

- Estimation of the potential glucose release from cellulose under simulated gastric and intestinal conditions.
- Report on the potential of controlled release technology for delivery of enzymes.
- Generate ideas for possible improvements to cellulases and recommendations for future experiments.

Assays of cellulase performance were designed using US Pharmacopoeia formulations of simulated gastric and intestinal fluids. Two commercial cellulase products derived from *Trichoderma reesei* and *Penicillium funiculosum* were chosen for study. Each of these products contains a mixture of cellulases and hemicellulases.

The stability of these cellulase products was first tested in simulated gastric fluid. Both products were rapidly inactivated in gastric fluid at pH 1.6 although certain individual enzymes were shown to be much more stable than the other components within the mixtures. By raising the pH of the simulated gastric fluid to 3.0 or above good stability of both cellulase products could be maintained over a 2 hour period. At the higher pH the pepsin present in the simulated gastric fluid is less active. It is feasible to raise the pH of the human stomach using antacids. The optimal pH for activity of the *Trichoderma* and *Penicillium* enzymes is 4.5 and raising the gastric pH to this level would maintain enzyme stability.

Without the use of antacids it will be necessary to develop enzymes with low pH optima and resistance to pepsin if cellulase activity in the stomach is desired. Alternatively, controlled release formulations would be required to protect cellulases during passage through the stomach and allow them to be released for activity in the intestines.

A potential benefit of low gastric pH might be that crystalline cellulose substrate would be modified to be more digestible by cellulase. This benefit would be lost if antacid were used to raise the gastric pH. However, tests demonstrated that treatment of Sigmacell crystalline cellulose with simulated gastric fluid at pH 1.6 did not render it more susceptible to hydrolysis by cellulase.

The stability of the two cellulase products was tested in simulated intestinal fluids at pH 5.0 or 7.0 over a 5.5 hour period. At pH 7 the *Trichoderma* product was inactivated, although not as rapidly as in simulated gastric fluid. At pH 5 inactivation was much less in intestinal fluid. The *Penicillium* cellulase product had higher stability than the *Trichoderma* product.

Genencor has considerable experience of formulation of enzymes on coated granules using fluidized bed technology and a report was prepared on this topic. A variety of controlled release coatings can be layered onto these granules. Enteric coatings have been shown to protect enzymes during passage through the low pH gastric environment and allow active enzyme release triggered by the higher pH intestinal environment. Other coatings allow for a time-dependent, rather than pH-dependent, release of enzyme. Products with a mixture of different enzyme granules could be devised to allow different release profiles. It would be possible for some cellulase to be released in the stomach and for further aliquots of enzyme to be released early and/or later in the intestines.

The hydrolysis of cellulose in simulated gastric and intestinal fluids was evaluated. For many of these studies a crystalline cellulose (Sigmacell) was used as substrate. The experimental design comprised addition of 10% w/v cellulose substrate to simulated gastric fluid with or without cellulase. After 2h incubation, the mixture was diluted and adjusted to match simulated intestinal fluid at pH 5, 6 or 7 and additional cellulase was added. Hydrolysis was continued for a further 24 hours. Throughout the experiment samples were taken and analyzed by HPLC for released glucose, cellobiose and cellotriose. A number of experiments of this type were conducted with *Trichoderma* or *Penicillium* cellulases and with variations in cellulase concentration, pH, etc. The overall conclusion of these studies was that approximately 30% of the cellulose could be converted to soluble sugars under the simulated gastric and intestinal conditions using high cellulase doses.

Some improved cellulase experimental products recently developed at Genencor were compared to the commercial enzyme products but none showed an improvement under these conditions.

It was of interest to determine what aspect of our experimental design limited hydrolysis to approximately 30% of the cellulosic substrate. Possibilities included the cellulase dose, the hydrolysis time, end product inhibition or the nature of the cellulosic substrate. Varying cellulase dosage showed that little advantage accrued if the dose was increased above 60 mg enzyme/g substrate. Hydrolysis of Sigmacell in simulated gastric or intestinal fluid was performed over an extended period of up to 8 days. Even over this period, which is obviously much longer than the human gastrointestinal residence time for food, only 50-60% conversion of the cellulose to soluble sugars was observed.

Other cellulosic substrates were also tested in hydrolysis experiments. A variety of commercially available food grade fibers were tested as substrates. For several of these it was possible to release 30 mg of soluble sugar from 100 mg substrate during an overnight digestion in simulated gastric fluid. Unfortunately, figures were not available for the composition of these substrates and the percentage by weight that was represented by cellulose. However, these food grade fiber substrates will be useful in the design of future animal or human trials. An experiment was performed with one of these food-grade substrates (wheat fiber) to simulate gastric (2 hours, pH 4.3 with pepsin) followed by intestinal (pH 6.0 with pancreatin) hydrolysis. Approximately 50% conversion of the substrate was possible under these conditions.

Almost 100% of phosphoric acid swollen cellulose (an amorphous, non-crystalline cellulose) could be converted to soluble sugars demonstrating that it was the crystalline form of Sigmacell that limited conversion to 30% and not end product inhibition. Of course, cellulose in the diet will not be in the form of amorphous cellulose but will be in crystalline form so further experiments to understand the limitation to crystalline cellulose (Sigmacell) hydrolysis were designed.

A series of experiments was performed in which Sigmacell was hydrolyzed by cellulase until the reaction rate slowed. At this point, we separated the supernatant containing enxyme with soluble sugars and the solid substrate, the solid substrate was washed and then either fresh enzyme was added to this partially digested substrate or the used enzyme was added to fresh substrate. In summary, these experiments showed that removal of supernatant from the substrate and addition of fresh enzyme ultimately allowed 100% conversion to soluble sugars. Thus, there does not appear to be a fraction of the substrate that is resistant to hydrolysis and would limit the extent of conversion in our earlier experiments. As the concentration of soluble sugars builds up in the supernatant the rate of substrate hydrolysis slows. However, this is not due to simple product inhibition of the enzymes because addition of the old supernatant containing the enzyme/sugar mixture to new solid substrate allows renewed hydrolysis, albeit at a slower rate.

The *Trichoderma* and *Penicillium* enzyme products used in the studies to this point are mixtures of number of individual enzymes including cellobiohydrolases that release cellobiose from the ends of cellulose chains, endoglucanases that cleave cellulose chains internally to create new free ends, and beta-glucosidases that hydrolyse cellobiose and short chain cellooligosaccharides to produce glucose. The synergistic action of these three enzyme types is required for efficient hydrolysis of cellulose. For human nutrition, an important criterion will be the extent to which cellulose is broken down completely to glucose, which can be utilized by humans as opposed to soluble cellooligosaccharides that cannot readily be utilized. The HPLC methods that we have used in this project to monitor conversion of cellulose allows separate quantification of glucose, cellobiose and cellotriose. In an attempt to improve the cellulase mixture we have purified *T. reesei* beta-glucosidase I and added this at varying dosage to the whole cellulase mixture produced by *Trichoderma*. The conversion of Sigmacell using these enzyme mixtures was studied. Addition of beta-glucosidase improved overall conversion of the cellulose substrate to soluble oligosaccharides slightly. A clear improvement could only be observed at very long incubation times and only a few percent improvement was apparent during 20h incubation of substrate and enzyme. However, it was also clear at all time points that elevated beta-glucosidase caused higher conversion to glucose with lower residual cellobiose in the supernatant.

Experiments were performed to determine if there are combinations of the major cellulase components (CBHI, CBHII, EGI and EGII) in the *Trichoderma* cellulase that are better at breaking down cellulose (pretreated corn stover) than the natural mix. To achieve this the enzyme products obtained from three different deleted strains of *Trichoderma* (EGI and EGII deleted, CBHI deleted or CBHI and CBHII deleted) were

mixed in various ratios. None of these enzyme mixtures was able to outperform the natural mix in terms of overall conversion to soluble sugars although some gave improved conversion to glucose and lower residual cellobiose.

The following overall conclusions were drawn from this project:

- 30% conversion of a 10% suspension of crystalline cellulosic substrate can be achieved under simulated gastric and intestinal conditions using a dose of enzyme of 60 mg per g of substrate. Thus, 100g of fiber containing 50g of cellulose could yield 16.5 g of glucose if 3g of cellulase were consumed.
- Up to 90% of cellulose hydrolysis is possible under simulated GI tract conditions depending upon the substrate. Further evaluation would require better *in vitro* and/or *in vivo* models of the GI tract with a more representative substrate containing hemicellulose and lignin in addition to cellulose.
- Buffering of the stomach contents (antacid) may allow early hydrolysis of cellulosics.
- There is potential for targeted delivery of enzymes and other functional components (e.g., peptides, probiotics, prebiotics, etc.) to the GI tract.
- There is potential for enhanced hydrolysis via a tailored enzyme mix and/or protein engineering to improve enzyme performance.
- There is potential for generation of or release of "functional" carbohydrates using enzyme treatment of food.

Meeting attendance

A project meeting was held at the Natick Army Center on September 14-16, 2004. The meeting was attended by Michael Ward, Kathleen Clarkson and Douglas Willrett of Genencor International.

The Intestinal Fortitude Workshop December 2, 2004 was attended by Kathleen Clarkson of Genencor International.

Publications and technical reports

Michael Ward, Lynn Tierney and Kathleen Clarkson. Feasibility Assessment for the Use of Cellulase in Biomass Conversion for Human Applications. Interim Progress Report 08/19/2003 to 12/31/2003, ARO.

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Report of Inventions

None